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A Kinetic Test for the Assay of the C1 Esterase-Inhibitor[1])

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Summary: The most satisfactory diagnostic procedure for hereditary angioneurotic oedema is the demonstration of low serum levels of C1 esterase-inhibitor. A modified method for the assay of this protein is described. It is based on the kinetic measurement of the C1 esterase-inhibitor when it inhibits the hydrolysis of N-acetyl-*L*-tyrosine-ethyl ester by C1 esterase. The relative C1 esterase-inhibitor concentration is based on the initial hydrolytic velocity, which can be evaluated from the pH change in a short time and within a small range. High reproducibility, cheap instrumentation and short time of analysis are some of the favorable aspects of this method in comparison with the 'end point titrimetric' method. Furthermore, this paper describes the mechanism of inhibition of C1 esterase by C1 esterase-inhibitor. The results are indicative of a non-competitive mechanism. The value of the *Michaelis-Menten* constant, K_m, is 0.017 ± 0.001 mol/l at 37 °C, in the optimum pH range 7.2–7.4. An estimate of K_I in arbitrary units is also given.

Kinetischer Test für die Bestimmung des C1-Esterase-Inhibitors

Zusammenfassung: Das zufriedenstellendste Verfahren zur Diagnostik des hereditären angioneurotischen Ödems ist der Nachweis der erniedrigten Konzentration des C1-Esterase-Inhibitors im Serum. Eine modifizierte Methode zur Bestimmung dieses Proteins wurde erarbeitet. Sie beruht auf der kinetischen Messung des C1-Esterase-Inhibitors, der die von C1-Esterase katalysierte Hydrolyse des Substrats N-Acetyl-*L*-tyrosin-ethylester hemmt. Die relative Konzentration des C1-Esterase-Inhibitors kann aus der Anfangsgeschwindigkeit der Hydrolyse, die in kurzer Zeit und innerhalb eines geringen Bereichs aus der pH-Zeitkurve berechnet wird, ermittelt werden. Hohe Reproduzierbarkeit, billige Ausrüstung und kurze Analysendauer sind einige wichtige Aspekte dieser Methode im Vergleich zur Endpunkt-Titrationsmethode. Ferner wird der Mechanismus der Hemmung der C1-Esterase durch C1-Esterase-Inhibitor beschrieben. Die Ergebnisse weisen auf einen nichtkompetitiven Mechanismus hin. Die *Michaelis-Menten*-Konstante K_m beträgt 0,017 ± 0,001 mol/l bei 37 °C im optimalen pH-Bereich von 7,2–7,4. Ein Schätzwert für die Inhibitorkonstante K_I in freigewählten Einheiten wird angegeben.

Introduction

Hereditary angioneurotic oedema is a well defined clinical syndrome characterized by a repeated occurrence of acute attacks of oedema in any organ, skin and mucosae of the upper respiratory and gastrointestinal tracts.

The most satisfactory diagnostic procedure for hereditary angioneurotic oedema is the demonstration of low serum levels of C1 esterase-inhibitor (1), which modulates the activation of the complement classical pathway. In its absence C1 activation proceeds autocatalytically and reacts with its natural substrates C4 and C2. The enzymatic assay of C1 esterase-inhibitor is to be preferred to the immunochemical method, since a variant of hereditary angioneurotic oedema is known, which is due to a functional deficiency of the inhibitor (2, 3, 4). The enzymatic measurement was performed by evaluating the esterolytic activity of the C1 esterase on the synthetic substrate, N-acetyl-*L*-tyrosine-ethyl ester,

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by microformol titration, as described by *Levy & Lepow* (5). Other authors (6,7) have subsequently modified this method based upon the hydrolytic property of C1 esterase on N-acetyl-*L*-tyrosine-ethyl ester. C1 esterase is added to the inhibitor, until hydrolysis is complete, the reaction being followed to the end point with a pH-stat automatic titrator.

This paper describes the kinetic mechanism of the inhibitory effect of the C1 esterase-inhibitor on the hydrolysis of N-acetyl-*L*-tyrosine-ethyl ester catalysed by C1 esterase. Furthermore, it presents a modified technique for the assay of C1 esterase-inhibitor. High reproducibility, cheap instrumentation and quick analysis are some of the favorable aspects of this method.

Materials

The reactor is stirred magnetically and thermostated by means of a water jacket at 37 ± 0.1 °C for 15 min. The pH is measured and registered with a microelectrode and a pH-meter (E 436 Metrohm Herisau).

Enzyme: purified C1 esterase (Cordis, Miami), activated with an equal volume of saline solution at 37 °C for 15 min. The amount of enzyme was expressed in terms of its activity. One unit (U) of C1 esterase is defined as the amount which liberates 1 μmol of acid per min (IUB), under the conditions defined below. The concentration of the activated solution of enzyme is 150 mU/l.

Substrate: N-acetyl-*L*-tyrosine-ethyl ester 1 mol/l in methyl cellosolve (2-methoxy ethanol).

Inhibitor: two types of inhibitor have been used: 1. pool of normal human sera of blood donors stored at -70 °C in a refrigerator (S10) with liquid nitrogen; this preservation is very important, owing to the easy degradation of C1 esterase-inhibitor, 2. purified C1 esterase-inhibitor (Cordis, Miami).

Method

A defined amount (1100–1200 μl) of saline solution is added to the reactor together with normal human serum (0–40 μl) or purified C1 esterase-inhibitor (0–120 μl); N-acetyl-*L*-tyrosine-ethyl ester 1 mol/l is slowly added until the final concentration (0.005–0.067 mol/l) is attained; during these operations the pH is maintained at the optimum value in the range 7.2–7.4 by adding small amounts of 0.02 mol/l NaOH. Finally, all the activated enzyme (300–400 μl) is added and the pH is registered. From the slopes of the pH/time curves the instantaneous velocity of the reaction, $d[H^+]/dt$, can be calculated. Reaction rates are determined with the test serum and with a pool of normal sera, and inhibition is expressed as a percentage of that observed with normal serum.

Results

Hydrolysis of the synthetic substrate (S) initiated by C1 esterase (E) was studied with normal human serum containing C1 esterase-inhibitor (I), and with a purified inhibitor. The results are illustrated in figures 1 and 4 for the normal human serum and in figure 2 for the purified C1 esterase-inhibitor.

As shown in figure 1 the amount of hydrogen ions freed, at given substrate and enzyme concentrations, is a linear function of time for a wide range of added amounts of normal human serum (0–40 μl). Identical results were obtained using scale dilutions of purified C1 esterase-inhibitor instead of normal human serum. The slope of the straight lines of figure 1 represents the instantaneous velocity of the hydrolytic reaction, $d[H^+]/dt$ [mol/l · s], which is effectively independent of time for the first few minutes of the reaction. A longer lasting autocatalytic effect of C1 esterase present in the normal

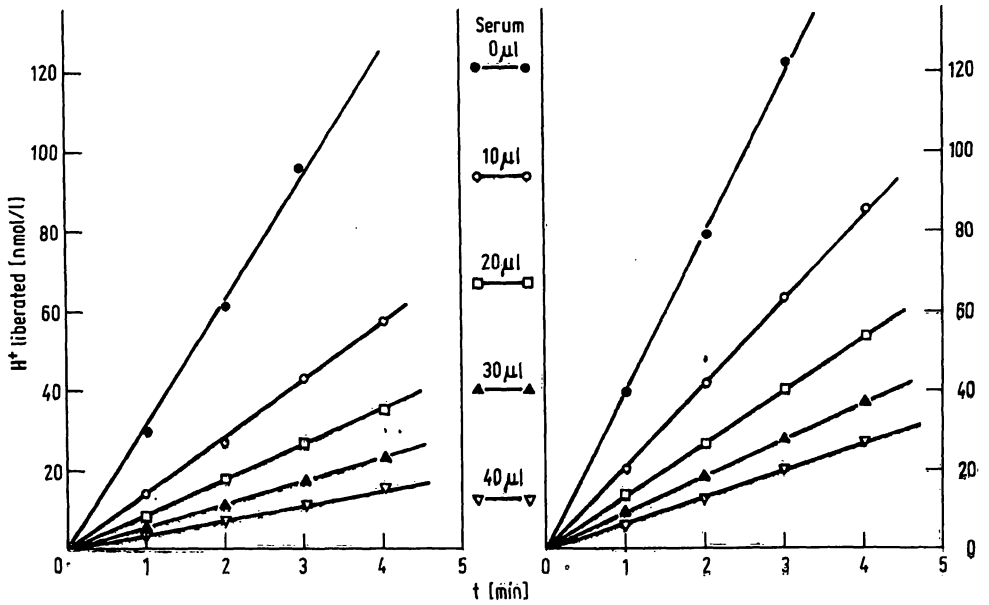

Fig. 1. Hydrolysis of the N-acetyl-*L*-tyrosine-ethyl ester 0.05 mol/l as a function of (t), at various amounts of normal human serum (μl), containing C1 esterase-inhibitor, by C1 esterase (left: C1 esterase=300 μl; right: C1 esterase=400 μl, corresponding to 30 and 40 mU/l in the final solutions, respectively).

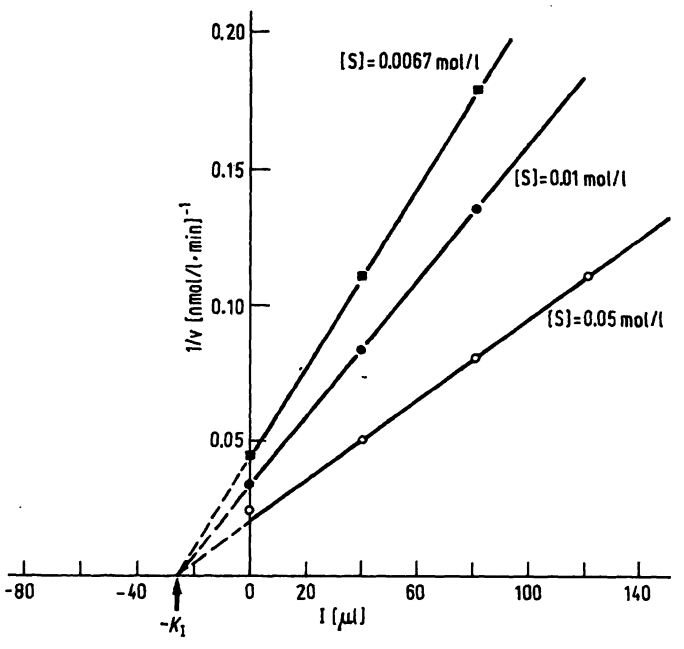

Fig. 2. Inverse of the rate of hydrolysis versus inhibitor (I=µl of purified C1 esterase-inhibitor) at various concentrations of substrate, for calculation of K_I. C1 esterase concentration is 40 mU/l.

human serum is to be expected (8,9). This effect, if present, is negligible in our experimental conditions, since no autoacceleration was detected, as clearly shown in figure 1. It should be noted that the rate of the hydrolytic reaction is proportional to the enzyme concentration (30–40 mU/l), whereas the presence of the inhibitor has a negative influence on the rate of hydrolysis.

The effect of the substrate concentration on the rate of hydrolysis was extensively studied with normal human serum and with or without purified C1 esterase-inhibitor. The double-reciprocal plot showed a linear dependence of 1/v on 1/[S], for $[S] \leqslant 0.025$ mol/l. When $0.025 < [S] < 0.05$ mol/l, the velocity attains a broad plateau and then decreases. This phenomenon is probably due to the difficulty in keeping the system homogeneous at high concentrations of the substrate. Similar results can be obtained by plotting the inverse velocity against scale amounts of purified C1 esterase-inhibitor at different concentrations of the substrate (fig. 2). Here again 1/v and I are linearly dependent and the slope is influenced by the concentration of S. It should be emphasized at this point that an inhibitor concentration cannot be expressed in terms of inhibited units per liter due to the reversibility of the equilibria involved. The amounts of inhibitor are expressed in terms of µl of inhibiting solutions; in fact figure 2 shows that 1/v is linearly correlated to I; consequently U is hyperbolically correlated to I. The equilibrium constant with the inhibitor, K_I, can be calculated in µl units by extrapolating the different lines onto the abscissa axis. On the other hand when analysing with normal human serum, we utilized a substrate concentration [S] = 0.05 mol/l, as suggested by *Haines & Lepow* (10). The relationship 1/v versus I is also valid at high substrate concentrations, as shown in figure 2; in this study a concentration of [S] = 0.025 mol/l gave approximately the same results.

The results are also reported in figure 3 in terms of the direct-linear plot of *Eisenthal & Cornish-Bowden* (11). This figure shows that the inhibition mechanism is non-competitive and provides an accurate estimation of K_m.

The linear correlation between 1/v and the amount of purified C1 esterase-inhibitor, expressed in µl, also holds when the inhibitor is present in the normal human serum (fig. 4). Samples of scale quantities of normal human serum (0–40 µl) were tested at an enzyme concentration corresponding to 40 mU/l. Figure 4 represents the calibration plot of the inhibition percentage in respect to pooled normal sera. Twenty normal subjects were tested and they showed a range of variability of 83–126% pooled normal sera ($\bar{x} \pm 2SD$). C1 esterase-inhibitor in the serum of a patient with hereditary angioneurotic oedema was 43%; it was 18% in serum when C1 esterase-inhibitor was inactivated by heating for 30 min at 59 °C. This latter value confirmed the fact that C1 esterase-inhibitor is thermolabile (12).

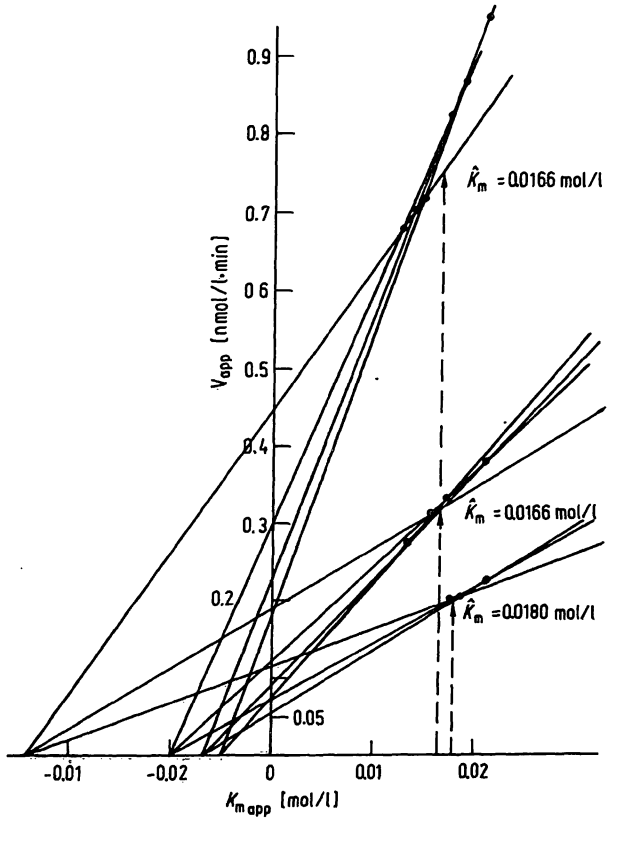

Fig. 3. Direct-linear plot of *Eisenthal & Cornish-Bowden*. Each intersection provides an estimate of V and K_m. The shifts of the intersections at the same value of K_m indicate a non-competitive mechanism.

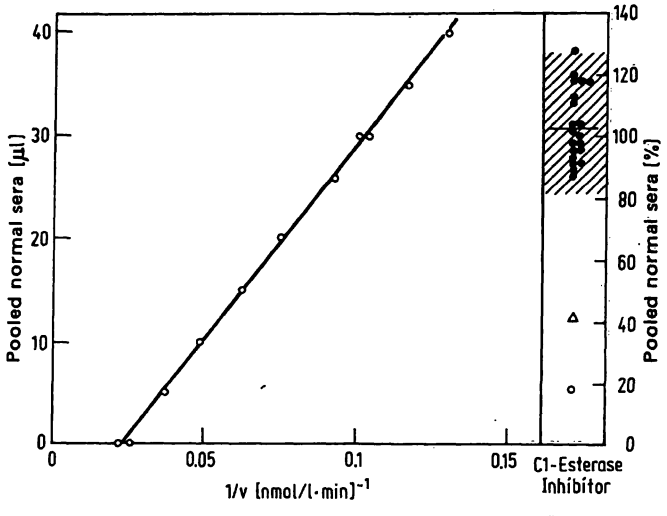

Fig. 4. Calibration plot of percentage inhibition with respect to pooled normal sera. The amount of C1 esterase-inhibitor (µl of pool of normal sera) as a function of 1/v. 1/v is the inverse of velocity of N-acetyl-*L*-tyrosine-ethyl-ester (0.05 mol/l) hydrolysis by C1 esterase (40 mU/l). The scale of % pooled normal sera is shown on the right, where 100 % corresponds to 30 µl; also the range of variability ($\bar{x}$ ± 2SD) determined from 20 blood donors (•); (△) patient with hereditary angioneurotic oedema; (○) human serum heated at 59 °C for 30 min.

Discussion

The experimental results compare well with the results reported by *Haines & Lepow* (10), who studied the enzyme kinetics in absence of the inhibitor.

Due to various factors in our system and the way the velocity was measured, the effect of pH on the rate of hydrolysis should be taken into consideration. In the experimental pH-range (7.2–7.4) the activity of the enzyme and the inhibitor are on a plateau (6.7–8.0). In fact, *Haines & Lepow* (10) have shown that when the pH is varied between 6.7 and 8.0, the C1 esterase activity towards N-acetyl-*L*-tyrosine-ethyl ester is optimal; *Pensky* et al (12) found a greater pH-range for the optimum of C1 esterase-inhibitor function. On the other hand, the results of this study showed that the pH had no significant effect on the system under the experimental conditions.

Concerning the relationship between the velocity of esterolytic reaction and the inhibitor concentration, the data obtained show that 1/v is linearly related to the µl of normal human serum or the µl of purified C1 esterase-inhibitor. This reaction can be expressed by the equation: $1/v = K_1 + K_2$ [I], which allows us to measure the inhibition, expressed as % pooled normal sera. The principle on which this assay is based derives from the mechanism of inhibition. In 1975 *Harpel & Cooper* (13) studied the molecular basis of the interaction between C1 esterase-inhibitor and C1 esterase. With SDS gel-acrylamide electrophoresis they saw that the enzyme-inhibitor complex was in equilibrium with the free enzyme and free inhibitor; no cleavage peptides of the C1 esterase-inhibitor were found as a consequence of the action of C1 esterase, suggesting that this was not the real substrate for the enzyme. However, these findings were not sufficient to determine the type of inhibition and the equilibrium present in this system. Although other authors (10, 12) had studied the kinetic characteristics of C1 esterase and some properties of C1 esterase-inhibitor, these aspects of the problem had not been taken into consideration. The enzymatic dosage suggested by them, which gave way to successive methods, was based on the general ability of C1 esterase-inhibitor to diminish the hydrolytic activity of the C1 esterase on N-acetyl-*L*-tyrosine-ethylester while the kinetic problem was not duly taken into consideration. The experiments carried out in our laboratories with purified C1 esterase-inhibitor have confirmed the results with human serum, and they have permitted the characterization of the mechanism of C1 esterase inhibition, and the determination of the *Michaelis-Menten* constant, K_m, and the constant of equilibrium with I, K_I. The results are indicative of a *non competitive mechanism of inhibition*, as clearly shown in the *Dixon* plot (fig. 2) as well as in the *Eisenthal & Cornish-Bowden* plots (fig. 3). With this direct-linear plot the value of K_m is easily calculated as the average of each series of points. The non-competitive mechanism is confirmed by the shift of the common intersection points at a practically constant value of K_m = 17 mmol/l. This value can be compared to 19 mmol/l, obtained by *Haines & Lepow* (10).

Based on non-competitive inhibition, a new kinetic assay is suggested in place of the previously reported method (8), which seems to be less rigorous and reproducible. The processes involved in the method reported by *Lachmann* et al. (8) are in fact complicated by the presence of the phosphate buffer, whose basic component (HPO_4^{2-}) reacts with the H^+ freed in the enzymatic reaction, when repeated amounts of C1 esterase are added. This implies that the 'end point' claimed by *Lachmann* et al. (8) is attained when enough C1 esterase is added to overcome the effect of HPO_4^{2-}, in addition to that of the inhibitor alone. It is worth noting that one should keep in mind the *exact quantity* of HPO_4^{2-} present. This factor has been explicitly considered by the authors. Moreover, the repeated sampling of C1 esterase contributes to the systematic errors, and it decreases the rapidity of the method by a factor of 5. In contrast, our method is highly reproducible as is shown in the calibration plot of figure 4, where the regression factor is 0.998. Furthermore our method is more economic, since it is not necessary to use a complicated pH-stat, but simply a pH-meter connected to a suitable recorder.

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